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Calcium pentosan polysulfate directly inhibits enzymatic activity of ADAMTS4 (aggrecanase-1) in osteoarthritic chondrocytes

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Abstract **Aggrecanases that include ADAMTS1, 4, 5, 8, 9 and 15 are considered to play key roles in aggrecan degradation in osteoarthritic cartilage. Here we demonstrate that calcium pentosan polysulfate (CaPPS) directly inhibits the aggrecanase activity of ADAMTS4 without affecting the mRNA expression of the ADAMTS species in interleukin-1α-stimulated osteoarthritic chondrocytes. Synthetic peptides corresponding to specific regions of the thrombospondin type 1 repeat, cysteine-rich or spacer domain of ADAMTS4 inhibit the binding to immobilized CaPPS. These data suggest that CaPPS could function as chondroprotective agent for the treatment of osteoarthritis by inhibition of ADAMTS4 through interaction with the C-terminal ancillary domain.**

Keywords: Osteoarthritis; ADAMTS4; Aggrecanase; Calcium pentosan polysulfate; Inhibition; Cartilage; Destruction

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis and characterized by loss of articular cartilage, which is ascribed mainly to proteolysis of the structural components of the extracellular matrix, i.e., proteoglycans and collagens [1]. Accumulated lines of evidence have indicated that aggrecanases play a central role in degradation of aggrecan, the major proteoglycan in cartilage, by cleaving at Glu-X bonds including the Glu^{392}–Ala^{393} bond [1,2]. These proteinases belong to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) gene family, and ADAMTS1, 4, 5, 8, 9 and 15 are known to exhibit the signature aggrecanase activity (cleavage at the Glu^{392}–Ala^{393} bond) [1,2]. Recent studies using ADAMTS4 (also called aggrecanase-1) and ADAMTS5 (aggrecanase-2) knockout mice have indicated that ADAMTS5 is responsible for cartilage destruction by aggrecan degradation in mice [3,4]. In human chondrocytes, however, ADAMTS4 is the aggrecanase induced by treatment with cytokines such as interleukin-1 (IL-1), whereas the ADAMTS5 expression is constitutive [5–7]. Our recent study on the expression of ADAMTS1, 4, 5, 8, 9 and 15 demonstrated that ADAMTS4 is selectively over-expressed in human OA cartilage and correlates directly with the extent of cartilage destruction, whereas ADAMTS5 is constitutively expressed [8]. Thus, it seems likely that ADAMTS4 is the major aggrecanase responsible for initial aggrecan proteolysis in human OA cartilage.

Calcium pentosan polysulfate (CaPPS) exhibits chondroprotective effects in experimental animal models of OA, and is used as a veterinary drug for animal OA [9,10]. It is also under clinical trials for human OA. Previous in vitro studies have shown to suppress release of aggrecan fragments from explant cultures of bovine articular cartilage [11,12]. However, these studies failed to describe the effects of CaPPS on mRNA expression of aggrecanases in OA chondrocytes, nor demonstrate the inhibitory activity of this agent on ADAMTS species. In this report, we provide the first evidence that CaPPS suppresses the aggrecanase activity through direct inhibition of ADAMTS4 by binding to its C-terminal ancillary domains but not the mRNA expression of the ADAMTS species.

2. Materials and methods

2.1. Materials

Recombinant human full-length ADAMTS4 and its C-terminus-truncated mutants, i.e., ΔSp lacking the spacer (Sp) domain, ΔCR/Sp lacking most of the cysteine-rich (CR) domain and the Sp domain and ΔTS/CR/Sp lacking the thrombospondin type 1 repeat (TS), CR and Sp domains were purified as described previously [13]. CaPPS and tissue inhibitor of metalloproteinases-3 (TIMP-3) were provided by Arthropharm (Sydney, New South Wales, Australia) and Daiichi Fine Chemical Co. Ltd. (Takaoka, Japan), respectively.

2.2. Chondrocyte cultures

Chondrocytes were isolated from articular cartilage obtained at arthroplasty from knee or hip joints of OA patients and cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum, ascorbic acid and antibiotics [14]. For the experimental use of the surgical samples, informed consent was obtained from the patients according to the Hospital ethical guidelines.

2.3. Inhibition of signature aggrecanase activity in cultured chondrocytes and cartilage with CaPPS

Chondrocytes were serum-starved in DMEM/F-12 containing 0.2% lactalbumin hydrolysate and treated with IL-1α (1 ng/ml) (Dainippon-Sumitomo Pharmaceutical Company Ltd., Osaka, Japan) and CaPPS (0, 0.1, 1 and 10 μg/ml) for 5 days in the presence of porcine aggrecan (100 μg/ml). The concentrated media were subjected to SDS–PAGE after deglycosylation and transferred onto PVDF membranes [13,15]. Aggrecanase activity was evaluated by immunoblotting with anti-$NITEGE^{392}$ neoepitope antibody (2 μg/ml) [13]. Similarly, explants of osteoarthritic cartilage (2 × 2 × 2 mm) were cultured for 3 days in 24-well plates

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(4 pieces/well) with 500 μl of DMEM/F-12 containing 0.2% lactalbumin hydrolysate in the absence or presence of IL-1α (1 ng/ml) and CaPPS (0, 0.1, 1 and 10 μg/ml). Cartilage tissues collected from each well were freeze-milled under liquid nitrogen into a fine powder, from which proteoglycans were extracted with 4 M guanidine hydrochloride, 10 mM EDTA, 1 M amino caproic acid, 50 mM sodium acetate, pH 6.8. Supernatants of the extracts were deglycosylated with chondroitinase ABC and keratanase (Seikagaku Corporation, Tokyo, Japan) and subjected to immunoblotting with anti-NITEGE^{392} neoepitope antibody.

2.4. Screening of mRNA expression of ADAMTS species by RT-PCR

Total RNA was extracted from the chondrocytes treated with or without IL-1α (1 ng/ml) and CaPPS (0, 0.1, 1 and 10 μg/ml) for 18 h and reverse-transcribed to cDNA using SuperScript II reverse transcriptase (Life Technologies, Rockville, MD). The cDNAs were amplified by PCR with primers specific to ADAMTS1, 4, 5, 8, 9 and 15 and housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as described previously [8].

2.5. Immunoblotting of ADAMTS4

Chondrocytes were cultured for 5 days under the serum-free conditions, and the concentrated media were subjected to SDS–PAGE under reduction and immunoblotted with anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) [8].

2.6. Inhibition of ADAMTS4 aggrecanase activity with CaPPS

Recombinant ADAMTS4 and ΔSp (10 nM) [13] were treated with CaPPS (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml) or TIMP-3 (100 nM) for 30 min at 37 °C and then incubated with aggrecan (2 mg/ml) for 16 h at 37 °C. After deglycosylation, the signature aggrecanase activity was monitored by immunoblotting using the anti-NITEGE^{392} neoepitope antibody [13,15]. Densitometrical analysis of the immunoreactive bands was performed by scanning densitometry using Scion Image (Scion Corp., Frederick, MD). IC_{50} values (concentration at 50% inhibition) were estimated by a scientific graphing and analytical software Origin 7.0 (Origin Lab Corp., Northampton, MA). We also carried out immunoblotting analysis for the above-mentioned samples using anti-SELE^{1564} neoepitope antibody (5 μg/ml) [16], which was kindly provided by Dr. Amanda J. Fosang (Department of Paediatrics and Murdoch Childrens Research Institute, University of Melbourne, Melbourne, Australia).

2.7. Binding of ADAMTS4 to immobilized CaPPS

Solid-phase binding assay was performed by incubating ^{125}I-labeled ADAMTS4, ΔSp, ΔCR/Sp, ΔTS/CR/Sp [13] or bovine serum albumin (BSA) in CaPPS-coated wells, which were prepared by reacting streptoavidin-coated microplates with 1 mg/ml biotinylated CaPPS, followed by washing with 50 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 and subsequent blocking with 3% Block-Ace (Dainippon-Sumitomo Pharmaceutical Company Ltd., Osaka, Japan). For inhibition study, the microplates were incubated with synthetic peptides (0.25, 0.5, 1 and 3 mg/ml) corresponding to heparin binding region in the TS domain of ADAMTS4 (GGWGPWGPWGD^{531}), the glycosaminoglycan binding region in the CR domain (GSKKKF-DKCM^{676}), a part in the CR and Sp domains (SFRKFRYG^{698}), a part in the Sp domain (LRRRPWAGRK^{837}) or RASCETP (a negative control) for 1 h at 23 °C prior to the binding assay. Reaction was continued by adding ^{125}I-labeled ADAMTS4 or BSA (2×10^5 cpm/well, ~10 ng/well each) for 24 h at 4 °C. Binding activity of ^{125}I-labeled ADAMTS4 species and BSA to CaPPS was determined by calculating the ratios (percentage) of radioactivity bound to CaPPS on the wells to total counts added using a γ-counter.

2.8. Statistical analysis

Comparisons against control were performed by Dunnett's multiple comparison test. *P* values less than 0.05 were considered significant.

3. Results and discussion

OA chondrocytes showed weak signature aggrecanase activity under non-stimulated conditions. The activity was increased in the chondrocytes treated with IL-1α, and it was decreased in a dose-dependent manner by the treatment with CaPPS (Fig. 1A). Similarly, CaPPS dose-dependently inhibited the signature aggrecanase activity in OA cartilage tissue, which was stimulated with IL-1α (Fig. 1B). To study the effect of CaPPS on the expression of aggrecanases, we examined the mRNA expression of ADAMTS1, 4, 5, 8, 9 and 15 in IL-1α-stimulated OA chondrocytes by RT-PCR. As shown in Fig. 2A, the expression of ADAMTS4 appeared to be stimulated with IL-1α, but ADAMTS1, ADAMTS5 and ADAMTS9 were constitutively expressed. The expression of ADAMTS15 was decreased after the IL-1α stimulation and ADAMTS8 was negligibly expressed before or after treatment with IL-1α. Importantly, the expression of all these ADAMTS species in IL-1α-stimulated chondrocytes was not changed by the treatment with different concentrations of CaPPS (Fig. 2A). Real-time PCR confirmed these data (data not shown). In accord with the mRNA data, the production level of ADAMTS4

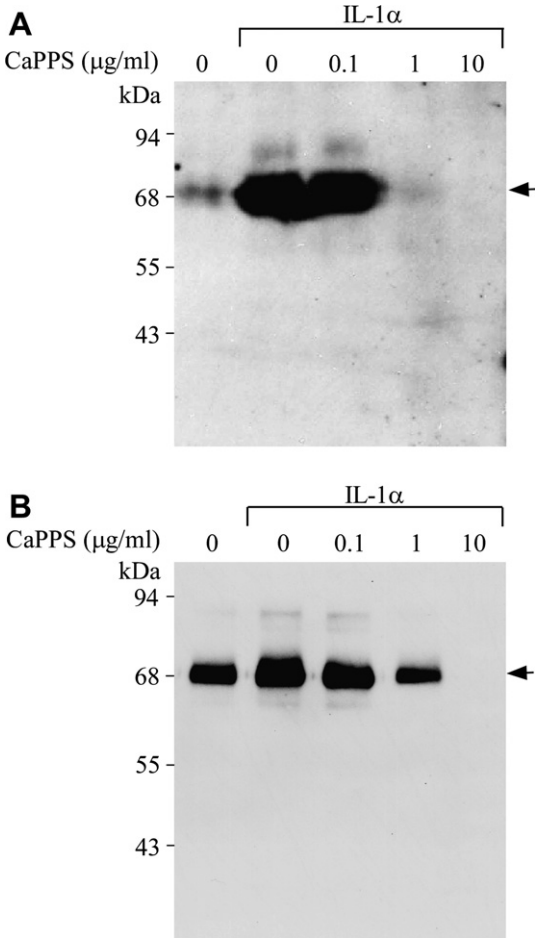

Fig. 1. Inhibition of signature aggrecanase activity in OA chondrocytes and cartilage with CaPPS. (A) OA chondrocytes were cultured in the absence or presence of IL-1α and CaPPS in serum-free DMEM/F-12 containing porcine aggrecan. Aggrecanase activity was evaluated by immunoblotting with anti-NITEGE^{392} neoepitope antibody. (B) Explants of OA cartilage were cultured in the absence or presence of IL-1α and CaPPS in serum-free DMEM/F-12 and aggrecanase activity was determined by immunoblotting of proteoglycans extracted from the cartilage. Arrows indicate the fragments immunoreactive with anti-NITEGE^{392} antibody.

protein in the culture media was increased by the IL-1α treatment, but CaPPS did not affect the level (Fig. 2B).

In the next experiment, we examined whether CaPPS directly inhibits the signature aggrecanase activity of recombinant full-length ADAMTS4 and its deletion mutant ΔSp, both of which exhibit aggrecanase activity [13]. As shown in Fig. 3A, density of the immunoreactive aggrecan fragment produced by the action of the ADAMTS4 species decreased in a dose-dependent manner by the CaPPS treatment. The densitometrical analysis of the bands indicated that IC_{50} values of ADAMTS4 and ΔSp are 0.2 and 2.4 μg/ml, respectively (Fig. 3B). When the effect of CaPPS on the ADAMTS4 aggrecanase activity against one of the chondroitin sulfate-2 sites was examined by immunoblotting with anti-SELE^{1564} neoepitope antibody, the activity was dose-dependently inhibited (Fig. 3C). These data demonstrate for the first time that CaPPS has no impact on the gene expression of ADAMTS1, 4, 5, 8, 9 and 15 in OA chondrocytes, but does directly inhibit the aggrecanase activity of purified recombinant ADAMTS4. We have previously shown that CaPPS enhances production of TIMP-3 at the posttranscriptional level in cultured rheumatoid synovial fibroblasts and OA chondrocytes [17]. Thus, CaPPS not only inhibits aggrecanase activity of ADAMTS4 produced by OA chondrocytes directly but also indirectly by increasing levels of its inhibitor, TIMP-3.

Solid-phase binding assay showed that among ^{125}I-labeled ADAMTS4, ΔSp, ΔCR/Sp and ΔTS/CR/Sp, only ADAMTS4 and ΔSp exhibit binding activity to CaPPS (data not shown). To further study the binding sites of ADAMTS4 to CaPPS, we carried out inhibition study of the binding of ADAMTS4 to CaPPS with synthetic peptides and found that the binding activity is inhibited by treatment with the peptides of GGWGPWGPWGD^{531} corresponding to a part of the TS domain, GSKKKFDKCM^{676} to that of the CR domain and

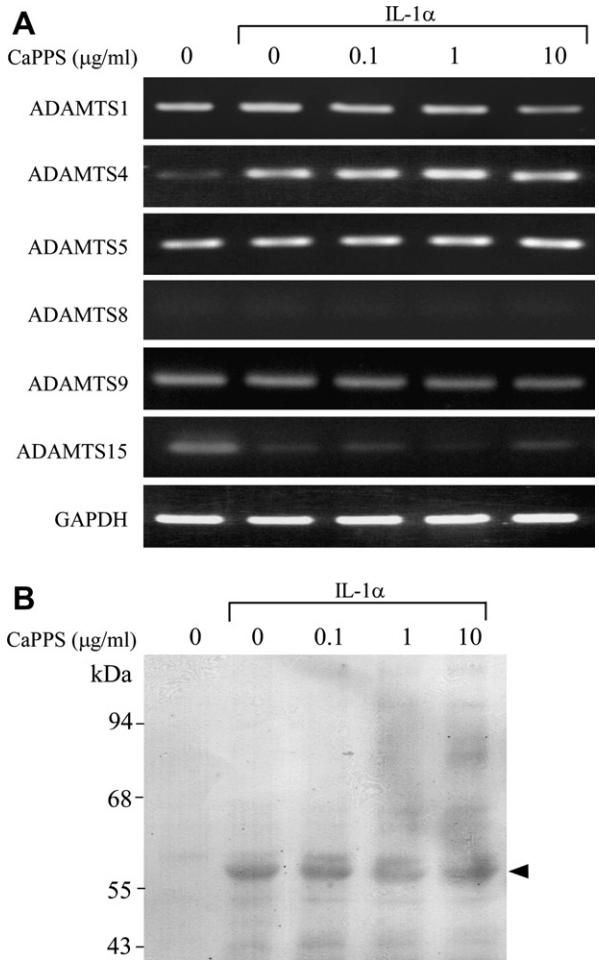

Fig. 2. Effects of CaPPS on the mRNA expression of ADAMTS species and production of ADAMTS4 protein in OA chondrocytes. (A) The mRNA expression of ADAMTS1, 4, 5, 8, 9 and 15 was examined by RT-PCR in the chondrocytes treated without or with IL-1α and CaPPS. GAPDH is shown as a control for loaded samples. (B) Immunoblotting analysis of the CaPPS effects on the ADAMTS4 expression. Chondrocytes were cultured in the absence or presence of IL-1α and CaPPS in serum-free DMEM/F-12 and conditioned media were subjected to immunoblotting using monoclonal antibody to ADAMTS4. Arrowhead indicates 58-kDa ADAMTS4.

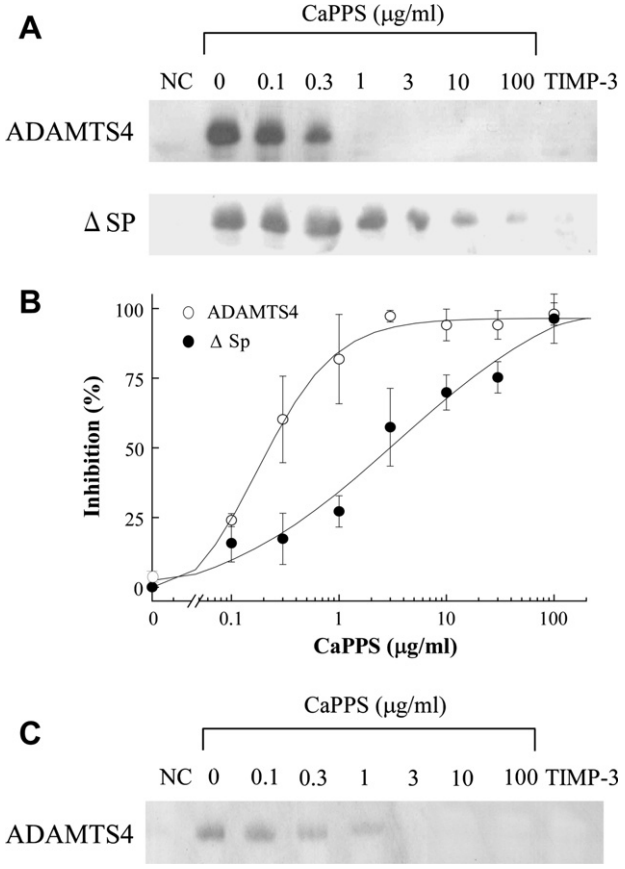

Fig. 3. Direct inhibition of ADAMTS4 aggrecanase activity by CaPPS. (A) Inhibition of signature aggrecanase activity of full-length ADAMTS4 and ΔSp with CaPPS. The recombinant ADAMTS4 species were incubated with buffer alone (NC), CaPPS or TIMP-3, and then reacted with aggrecan. After deglycosylation of the aggrecan samples, they were subjected to immunoblotting with anti-NITEGE^{392} neoepitope antibody. (B) Inhibition curves of the ADAMTS4 activity with CaPPS. Relative inhibition (%) of the signature aggrecanase activity of ADAMTS4 and ΔSp with CaPPS was calculated by densitometrical analysis of the bands. (C) Inhibition of the ADAMTS4 aggrecanase activity to the chondroitin sulfate-2 site with CaPPS. ADAMTS4 incubated with buffer alone (NC), CaPPS or TIMP-3 was reacted with aggrecan, and then the samples were subjected to immunoblotting with anti-SELE^{1564} neoepitope antibody.

LRRRPWAGRK^{837} to that of the Sp domain of ADAMTS4 (Fig. 4). However, no inhibition was observed with SFRK-FRYG^{698} corresponding to a part of the CR and Sp domains or RASCETP (a negative control). These data suggest that ADAMTS4 interacts with CaPPS at three sites of the TS, CR and Sp domains.

TIMP-3 is considered to be the physiological inhibitor of ADAMTS4 [15,18], and it is predictable that TIMP-3 inhibits the activity of ADAMTS4 by binding to the catalytic site. However, accumulated lines of evidence have demonstrated that the non-catalytic C-terminal ancillary domains including TS, CR and Sp domains play key roles in modulation of the ADAMTS4 aggrecanase activity [13,19–21]. The binding of the TS and Sp domains to aggrecan glycosaminoglycans is known to enhance TIMP-3 inhibition of ADAMTS4 activity [19], and we have previously demonstrated that fibronectin inhibits ADAMTS4 aggrecanase activity through its interaction with the Sp domain [13]. The present results showing the binding between CaPPS and the TS, CR and Sp domains strongly suggest that the inhibition of ADAMTS4 by CaPPS is ascribed to the hindrance of access of ADAMTS4 to aggrecan, since these domains are required for the aggrecanase activity of ADAMTS4 [13,21,22].

Since CaPPS is a heparin-like substance, heparin would also be expected to have inhibitory activity to ADAMTS4. Indeed, a recent study has shown that heparin inhibits the aggrecanase activity of the full-length ADAMTS4 through binding to the Sp domain [21]. However, the IC_{50} value of CaPPS to ADAMTS4 (0.2 μg/ml) is ~23-fold lower than that of heparin (4.5 μg/ml) [21], and CaPPS, but not heparin, can inhibit the aggrecanase activity of ΔSp through the interaction with the CR and TS domain. ADAMTS4 is present mainly in a C-terminus-truncated form, i.e. ΔSp, in pathological tissues including human cartilage and synovial tissue [8,22], which may be elaborated by the action of matrix metalloproteinases [23]. Thus, once ADAMTS4 is processed to ΔSp, neither fibronectin nor heparin can block the aggrecanase activity of ΔSp, but CaPPS is still able to achieve inhibition. Pharmacokinetic studies in several species have shown that within 4 h of intramuscular injections of 2–3 mg/kg of CaPPS plasma levels of 4–5 μg/ml are generally observed [9]. An equine study also revealed that an intramuscular dosage of 2 mg/kg CaPPS produced synovial fluid levels of 1–2 μg/ml over the same time period [24]. These studies indicate that the concentration of CaPPS obtained in synovial joints after administration of the recommended therapeutic dose for the treatment of OA is within the range that could achieve inhibition of ADAMTS4 activity in this compartment.

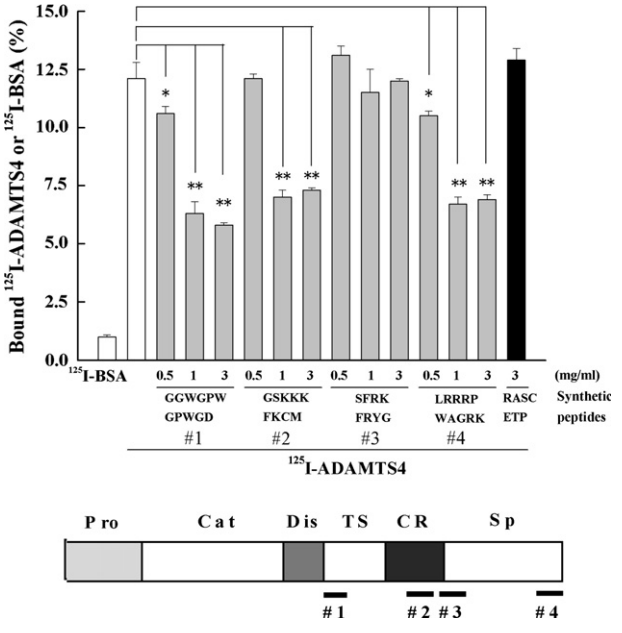

Fig. 4. Inhibition of the ADAMTS4 binding to immobilized CaPPS with synthetic peptides. Microplates coated with CaPPS were incubated with ^{125}I-labeled BSA or ADAMTS4 after incubation of the microplates without or with the synthetic peptides corresponding to the parts of the TS, CR or Sp domain of ADAMTS4 (see a lower panel showing schematic presentation of the ADAMTS4 domains) or RASCETP (a negative control). The radioactivity bound to CaPPS was counted using a γ-counter. Error bars indicate S.E. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$.

In animal models of OA, CaPPS has been shown to preserve cartilage integrity, but it also exhibited other pharmacological actions which could contribute to its chondroprotective activity [9,10]. Clinically, CaPPS [9] and the corresponding sodium salt, NaPPS [25] have been shown to significantly improve the symptoms and functional index of joints in a double blind placebo controlled clinical trials with OA patients. Although the observed improvement in joint symptoms may not reflect any beneficial of CaPPS on joint articular cartilage in the short time frame used in these clinical trials, the present observations of the ability of this agent to inhibit ADAMTS4 and increasing TIMP-3 production [17] lend further support to the assignment of this agent to the class of disease modifying drugs for OA. However, additional longer-term clinical and cartilage imaging studies will be required to confirm this assignment.

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